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The accumulation of eosinophils in lung tissue is a hallmark of asthma, and it is believed that eosinophils play a crucial pathogenic role in allergic inflammation. Prostaglandin (PG) E₂ exerts anti-inflammatory and bronchoprotective mechanisms in asthma, but the underlying mechanisms have remained unclear. In this study we show that PGE₂ potently inhibits the chemotaxis of purified human eosinophils toward eotaxin, PGD₂, and C5a. Activated monocytes similarly attenuated eosinophil migration, and this was reversed after pretreatment of the monocytes with a cyclooxygenase inhibitor. The selective E-prostanoid (EP) 2 receptor agonist butaprost mimicked the inhibitory effect of PGE₂ on eosinophil migration, whereas an EP2 antagonist completely prevented this effect. Butaprost, and also PGE₂, inhibited the C5a-induced degranulation of eosinophils. Moreover, selective kinase inhibitors revealed that the inhibitory effect of PGE₂ on eosinophil migration depended upon activation of PI3K and protein kinase C, but not cAMP. In animal models, the EP2 agonist butaprost inhibited the rapid mobilization of eosinophils from bone marrow of the in situ perfused guinea pig hind limb and prevented the allergen-induced bronchial accumulation of eosinophils in OVA-sensitized mice. Immunostaining showed that human eosinophils express EP2 receptors and that EP2 receptor expression in the murine lungs is prominent in airway epithelium and, after allergen challenge, in peribronchial infiltrating leukocytes. In summary, these data show that EP2 receptor agonists potently inhibit eosinophil trafficking and activation and might hence be a useful therapeutic option in eosinophilic diseases. 


Eosinophils are important effector cells in allergic diseases, particularly in late-phase reactions, because they release bronchoconstrictor mediators such as leukotriene C₄ and other chemoattractants that cause further influx of inflammatory cells into the tissue (1). Eosinophils produce proinflammatory cytokines and growth factors, including immunoregulatory Th2-type cytokines (2–4). Mucosal damage in asthma is associated with cytokotic mediators that are released by activated eosinophils, and it has been shown that asthmatic patients who received treatment based on eosinophil counts in sputum had significantly fewer severe asthma exacerbations than patients treated according to standard management therapy (5). Moreover, animal studies have also shown that genetically modified mice lacking eosinophils are protected against allergen-induced lung injury and asthma (6, 7).

A wide range of agonists can stimulate the accumulation of eosinophils, in particular those of the CC chemokine family, which are acting through the chemokine receptor CCR3 (8). In addition, activated complement factors such as C5a, leukotrienes, 5-oxo-6,8,11,14-eicosatetraenoic acid, and platelet-activating factor are also potent chemoattractants for eosinophils (9–11). Lately, a cyclooxygenase (COX)-derived mediator of activated mast cells, prostaglandin (PG) D₂, and several of its metabolites have been revealed to potentiate eosinophil chemotaxis (12, 13).

PGE₂ is the predominant cyclooxygenase product of airway macrophages, epithelial cells, and smooth muscle cells and is regarded as a potent inflammatory mediator due to its effects on vasoconstriction, vascular permeability, and nociception; however, the role of PGE₂ in allergic inflammation is unclear. In the asthmatic lung, PGE₂ affects both airway smooth muscle and the inflammatory process; PGE₂ causes bronchial relaxation (14) and inhibits allergen induced bronchoconstriction (15), but it may also provoke bronchoconstriction responses in some individuals (16) because of activation of C fibers and reflex cholinergic pathways (17). Moreover, PGE₂ can induce cough directly and sensitize the cough receptor (18). In humans and rats, PGE₂ reduces allergen-induced airway eosinophilia (19, 20), abrogates eosinophil accumulation after passive cutaneous anaphylaxis in the guinea pig (21), and protects against bleomycin-induced pulmonary fibrosis in mice (22). Conversely, eosinophil influx is exaggerated in COX-1 or COX-2 knockout mice (23, 24) and also in mice treated with selective COX-1 or COX-2 inhibitors (25). Decreased airway mucosal PGE₂ production during airway obstruction has been found in equine asthma. 

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4 Abbreviations used in this paper: COX, cyclooxygenase; BAL, bronchoalveolar lavage; CRTH2, chemoattractant receptor homologous molecules of Th2 cells; DM, demethylated; EP, E-prostanoid; EPO, eosinophil peroxidase; PG, prostaglandin; PKA, protein kinase A; PKC, protein kinase C.

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asthma (26). At the cellular level, PGE₂ has been found to attenuate IgE-dependent degranulation of eosinophils and leukotriene biosynthesis. In contrast, PGE₂ acts as a chemoattractant for immature murine mast cells derived from bone marrow (27). Moreover, PGE₂ may favor Th2 responses over those of Th1 and promote the isotype switch to IgE (28).

Taken together, the role of PGE₂ in allergic disease is not unequivocal, but both animal and human studies suggest that PGE₂ has the potential of limiting the allergen-induced inflammatory response. However, the underlying mechanisms of this effect and, hence, also the therapeutic usefulness of this approach still needs to be clarified. In this study we report that PGE₂ has a direct inhibitory effect on the migration of human eosinophils and that this effect is mediated by E-prostanoid (EP) 2 receptors. We further show in animal models that EP2 agonists potently inhibit the release of eosinophils from the bone marrow and the allergen-induced accumulation of eosinophils in the lung, thus pointing to EP2 receptors as therapeutic targets in allergic diseases.

Materials and Methods

Chemicals

Table I summarizes the pharmacological compounds used in this study and their target selectivity.

<table>
<thead>
<tr>
<th>Name</th>
<th>Selectivity</th>
<th>Activity</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH-6809</td>
<td>EP2 = EP1 &gt;</td>
<td>Antagonist</td>
<td>57</td>
</tr>
<tr>
<td>Butaprost</td>
<td>EP2</td>
<td>Agonist</td>
<td>57</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>PKC</td>
<td>Inhibitor</td>
<td>58</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>COX-1 = COX-2</td>
<td>Inhibitor</td>
<td>59</td>
</tr>
<tr>
<td>H-89</td>
<td>PKA</td>
<td>Inhibitor</td>
<td>60/61</td>
</tr>
<tr>
<td>ONO-24,8</td>
<td>EP3</td>
<td>Agonist</td>
<td>62</td>
</tr>
<tr>
<td>ONO-AE-329</td>
<td>EP4</td>
<td>Agonist</td>
<td>63</td>
</tr>
<tr>
<td>ONO-AE-3-208</td>
<td>EP4</td>
<td>Agonist</td>
<td>64</td>
</tr>
<tr>
<td>PMA</td>
<td>PGF2α</td>
<td>Agonist</td>
<td>65</td>
</tr>
<tr>
<td>SB-20190</td>
<td>P38 MAPK</td>
<td>Inhibitor</td>
<td>11</td>
</tr>
<tr>
<td>SC-51089</td>
<td>EP1</td>
<td>Antagonist</td>
<td>57</td>
</tr>
<tr>
<td>SQ-22536</td>
<td>Adenylyl cyclase</td>
<td>Inhibitor</td>
<td>60</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Protein kinases</td>
<td>Inhibitor</td>
<td>65</td>
</tr>
</tbody>
</table>

*DP is D-prostanoid receptor.

**PGE₂**

Preparation of human leukocytes

Blood was sampled from healthy volunteers according to a protocol approved by the Ethics Committee of the Medical University of Graz (Graz, Austria). All volunteers provided informed consent. Blood samples were processed as described previously (29, 30). In brief, platelet-rich plasma was removed by centrifugation of citrated whole blood, after which erythrocytes and platelets were removed by dextran sedimentation. Preparations of polymorphonuclear leukocytes, containing eosinophils and neutrophils, and PBMC, including monocytes, basophils, and lymphocytes, were made by Histopaque gradients (31). For chemotaxis, eosinophils were further purified from polymorphonuclear populations by negative magnetic selection using an Ab mixture (CD2, CD14, CD16, CD19, CD56, and glycoplin A) and colloidal magnetic particles from StemCell Technologies. Resulting populations of eosinophils were typically >97%, with the majority of contaminating cells being neutrophils.

Culture of human peripheral blood monocytes

PBMC were resuspended in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (20 mM), nonessential amino acids, HEPES (50 mM) and sodium pyruvate (10 mM) at 6.7 × 10⁶/ml. Aliquots were placed into 6.5-mm Transwell inserts with 5-μm polycarbonate filters (Corning) in 24-well culture plates and human AB serum was added. After 120 min of incubation at 37°C (5% CO₂) nonadherent cells were removed, which resulted in 10⁵ monocytes per well and a purity of 95% (32).

Chemotaxis

Purified eosinophils were suspended in assay buffer at 2 × 10⁶/ml, and 50 μl of the suspension was placed onto the top of a 48-well micro-Bronchial chemotaxis chamber with a 5-μm pore-size polycarbonate filter (NeuroProbe) and 30 μl of agonists was placed in the bottom wells of the plate. Baseline migration was determined in wells containing only assay buffer. The plates were incubated at 37°C in a humidified incubator for 1 h, and the membrane was carefully removed. Cells that had migrated to the lower chamber were enumerated by flow cytometric counting for 30 s, as described previously (30).

Transwell chemotaxis system (33) was used to assay the transmigration of eosinophils through layers of cultured adherent monocytes. Purified eosinophils were suspended in assay buffer at 2 × 10⁶/ml, and 100 μl of the suspension were placed onto the top of 5-μm polypolylysine-coated 6.5-mm Transwell inserts containing adherent and overnight-cultured monocytes. The inserts were placed into 24-well culture plates containing 600 μl of buffer or chemotactic buffer in the bottom well. The plates were incubated at 37°C in a humidified incubator for 1 h, and eosinophils that had migrated to the bottom wells were enumerated by flow cytometry.

Radioimmunoassay

Immunoreactive PGE₂ in the supernatants of overnight-cultured monocytes was determined as described previously (34) using [5,6,8,11,12,14,15-(3H)]PGE₂ as tracer and synthetic PGE₂ as standard. Charcoal was used to separate the free and Ab-bound fractions. The detection limit (defined as 10% inhibition of binding of tracer to antibody) was 5.5 pg.

Eosinophil degranulation

To determine the release of eosinophil peroxidase (EPO) from human eosinophils, cells were resuspended in assay buffer at 1 × 10⁶ cells/ml, mixed with cytocalasin B (10 μg/ml), and 50-μl aliquots were loaded into the wells of a 96-well microplate. Cells were stimulated with 20 μl of various concentrations of C5a for 20 min at 37°C. Thereafter, 60 μl of H₂O₂ (1 mM) were added to each well to start the peroxidase reaction. To detect the reaction, 70 μl of 2.8 mM tetramethylbenzidine was used. Following incubation for 1 min at room temperature, the peroxidase reaction and the color development were terminated with 4 M acetic acid (35). Microplates were analyzed on a bench reader at a wavelength of 630 nm. Data were expressed as the percentage of the maximal control response (C₅a at 300 nM).

In situ perfusion of the guinea pig hind limb

The guinea pig hind limb was perfused as previously described (29, 33). The external iliac artery and vein were exposed and the caudal abdominal artery, superficial ileal circumflex artery, and pudendopelvicartery.
trunk along with their satellite veins were ligated. Polyethylene cannulas (0.8-mm outside diameter) were inserted into the external iliac artery and vein. Modified Krebs-Ringer bicarbonate buffer (gassed with 95% O2 and 5% CO2 at 37°C) was infused at 4 ml/min via the arterial cannula and removed from the venous cannula using a peristaltic pump. After an equilibration period of 20 min, the perfusate fractions were collected every 10 min and centrifuged at 3000 rpm for 10 min. The cell pellet was resuspended in Kimura’s stain and nucleated leukocytes and Kimura-positive eosinophils were counted in a Neubauer hemacytometer (36).

Murine model of allergic lung inflammation

The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science. Male BALB/c mice, 6- to 8-wk old, were immunized with i.p. injections of 20 µg of OVA adsorbed to 3.5 mg of Al(OH)3 on days 0 and 7. Three groups of mice were studied. The control group did not receive any further treatment until the end of the experiment. The second and third groups of mice were challenged by an aerosol of 2% (w/v) of OVA in saline for 30 min on days 21, 23, and 25. Additionally, these groups were treated with vehicle or PGE2 (100 µg of 5% (v/v) ethanol) and group 3 was treated with 50 µg of butaprost. On day 27, mice were sacrificed by an overdose of pentobarbital sodium for the analysis of leukocytes in peripheral blood and bronchoalveolar lavage (BAL) fluid. Blood was sampled by cardiac puncture. BAL was performed with 3 x 1 ml of Ca2+- and Mg2+-free PBS supplemented with 0.1 mM EDTA. Eosinophils in blood and BAL were recognized by flow cytometry as highly granular cells (high side scatter) expressing the eotaxin receptor CCR3 (37).

Immunostaining of EP2 receptors

Mice were deeply anesthetized with sodium pentobarbital (60 mg/kg; i.p.), the lungs were perfused via the lung artery with 0.1 M PBS (pH 7.4) followed by 4% buffered paraformaldehyde. The lungs were dissected, postfixed for 48 h, and embedded in paraffin. Five-micrometer sections were deparaffinized, Ag retrieval was performed in 10 mmol/L sodium citrate solution (pH 6.0) (10 min at 600 watts in a microwave oven), and endogenous peroxidase was blocked with 0.3% H2O2 in PBS containing 0.1% Triton X-100. To reduce nonspecific background, sections were incubated in blocking solution (Vectastain ABC Kit). The samples were incubated overnight with a polyclonal EP2 Ab (diluted 1/100) or, to test for specific staining, with preabsorbed Ab (Ab preabsorbed with a 5-fold amount of the corresponding peptide Ag) at 4°C overnight. After washing, the bound Ab was detected using the Vectastain ABC kit with diaminobenzidine as the chromogen and counterstained with methyl green. Sections were visually examined with an Olympus IX70 microscope (Olympus) and an Olympus U Plan Apo 40/0.8 lens. Photographs were taken with an Olympus DP50 camera (2776 x 2074 pixels) and further processed with CELL® software (Olympus) for additional white balance, contrast, and brightness adjustments.

EP2 receptors on human peripheral blood eosinophils were quantified by indirect immunofluorescence flow cytometry. Because the EP2 Ab is directed against the C terminus of the EP2 receptor, aliquots of

FIGURE 1. PGE2 decreases the chemotactic responsiveness of human eosinophils to chemoattractants but has no effect on neutrophil migration. A–C, Purified human eosinophils were mixed with vehicle or PGE2 (10 and 30 nM), loaded into the top wells of a micro-Boyden chamber, and allowed to migrate toward PGD2 (A), eotaxin (B), and C5a (C). D, Purified human eosinophils were mixed with vehicle or increasing concentrations of PGE2 (1–100 nM) and allowed to migrate toward eotaxin (1 nM), C5a (1 nM) or PGD2 (10 nM) in the bottom wells. E and F, Neutrophils were mixed with vehicle or PGE2 (10 and 30 nM), and allowed to migrate toward IL-8 or C5a, respectively. Chemotaxis was expressed as a percentage of the maximum response to the respective control stimulus (i.e., chemoattractant in the bottom well only). Data are shown as mean ± S.E.; n = 4–6. *p < 0.05 PGE2 vs vehicle.
isolated eosinophils were first permeabilized with Fix&Perm solution (An Der Grub Bio Research) for 15 min at room temperature. Samples were then treated with the following consecutive Abs for 30 min each on ice with appropriate washing steps in between: 1 mg/ml human IgG to block Fc receptors; 20 μg/ml polyclonal EP2 Ab or preabsorbed Ab (EP2 Ab incubated with a 5-fold amount of the corresponding peptide immunogen); and 4 μg/ml anti-rabbit IgG secondary Ab conjugated with Alexa Fluor-488. After adding the fixative solution the cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis
Data are shown as the mean ± S.E. Statistical comparisons of groups were performed using two-way ANOVA for repeated measurements or Wilcoxon signed rank test. Probability values of \( p < 0.05 \) were considered as statistically significant.

Results

**PGE\(_2\) inhibits the migration of human eosinophils**

To assess the effects of PGE\(_2\) on chemoattractant-induced eosinophil chemotaxis, purified human eosinophils were mixed with vehicle or two dilutions of PGE\(_2\) (10 and 30 nM) for 5 min at room temperature and were allowed to migrate for 60 min in a micro-Boyden chemotaxis chamber toward serial dilutions of PGD\(_2\) (1–300 nM), the chemokine eotaxin (0.01–10 nM), or C5a (0.1 nM-30 nM) placed in the bottom wells. Migrated cells in the bottom wells were then enumerated by flow cytometry. Fig. 1 demonstrates that PGE\(_2\) potently inhibited the migration of human eosinophils toward eotaxin, PGD\(_2\), and C5a, while at the same concentrations the migration of neutrophils toward serial dilutions (0.1–10 nM) of IL-8 or C5a was not attenuated by PGE\(_2\) (10 and 30 nM; Fig. 1, E and F). In all experiments, the treatment of eosinophils with PGE\(_2\) was without effect on spontaneous migration in the absence of a chemoattractant (data not shown).

In additional experiments we investigated the concentration-effect relationship of the PGE\(_2\)-induced inhibition of eosinophil migration. To this end, purified human eosinophils were mixed with vehicle or serial dilutions of PGE\(_2\) (3–100 nM), and the cells were allowed to migrate toward single concentrations of eotaxin, C5a, or PGD\(_2\) in the bottom wells for 60 min. The concentrations of eotaxin (1 nM), C5a (1 nM), and PGD\(_2\) (10 nM) used were selected so that they induced chemotaxis of similar magnitude. In keeping with the results from the first experiments, PGE\(_2\) concentration dependently depressed the chemotactic responses of eosinophils, with maximal inhibition being observed with 100 nM PGE\(_2\).

Activated monocytes attenuate eosinophil migration via the release of PGE\(_2\)

These data suggested that PGE\(_2\) is a selective and potent inhibitor of eosinophil migration and activation. We next investigated whether endogenous PGE\(_2\), released in a paracrine manner, was involved in the regulation of eosinophil recruitment. In detail, we investigated how activated monocytes, which are a rich source of PGE\(_2\) and are constituents of the inflammatory cell infiltrate in asthma, could modulate eosinophil function. To this end, peripheral blood monocytes were isolated and cultured overnight on
Transwell filters in the presence or absence of 1 ng/ml LPS (*Escherichia coli* serotype 055:B5), and the migration of eosinophils through the monocyte layer toward eotaxin (1 nM) was determined. As compared with untreated monocytes, the migration of eosinophils in the presence of activated monocytes was reduced by 50% (Fig. 2C). Pretreatment of monocytes with the COX inhibitor diclofenac (1 μM) 20 min before exposure to LPS completely restored eosinophil responsiveness to eotaxin (Fig. 2C). Similarly, the low molecular fraction (<1 kDa) of the supernatants of LPS-activated monocytes, purified by centrifugation over 1-kDa filters (Pall Microsep), significantly attenuated the chemotaxis of eosinophils toward eotaxin (p < 0.05), whereas supernatants from monocytes that had been treated with diclofenac plus LPS displayed no inhibitory effect (data not shown; n = 6). These observations were mirrored by increased amounts of PGE2 released into the supernatants in the presence of LPS and by diclofenac decreasing PGE2 production below baseline levels (Fig. 2D). This suggested that a COX product, most likely PGE2, was released by activated monocytes and accounted for the inhibition of eosinophil migration.

**PGE2 inhibits eosinophil function via PI3K and PKC**

Elevation of intracellular cAMP has been suggested to attenuate leukocyte function and, in fact, PGE2 is an effective activator of adenyl cyclase via EP2 and EP4 receptors coupled to G proteins. To investigate the involvement of the adenyl cyclase/protein kinase A (PKA) pathway in the inhibitory action of PGE2 on eosinophil chemotaxis, purified human eosinophils were pretreated with vehicle, the adenyl cyclase inhibitor SQ-22536 (10 μM), or the PKA inhibitor H-89 (1 μM) at 37°C for 30 min and then mixed with 30 nM PGE2. Cells were loaded into the top wells of a micro-Boyden chamber and allowed to migrate toward eotaxin (1 nM; A and B) or PGD2 (30 nM; C). The data shown in B are from the same experiments as in A, but were normalized to the response in the presence of the respective inhibitor but in the absence of PGE2. Data are the mean ± SE; n = 5–12; n.d., not determined; *, p < 0.05 as indicated.

**FIGURE 3.** Inhibition of adenyl cyclase or protein kinase A does not prevent the PGE2-induced inhibition of eosinophil chemotaxis. Purified eosinophils were pretreated with vehicle, the adenyl cyclase inhibitor SQ-22536 (10 μM), or the protein kinase inhibitor H-89 (1 μM) at 37°C for 30 min and then mixed with 30 nM PGE2. Cells were loaded into the top wells of a micro-Boyden chamber and allowed to migrate toward eotaxin (1 nM; A and B) or PGD2 (30 nM; C). The data shown in B are from the same experiments as in A, but were normalized to the response in the presence of the respective inhibitor but in the absence of PGE2. Data are the mean ± SE; n = 5–12; n.d., not determined; *, p < 0.05 as indicated.

**FIGURE 4.** Inhibition of PI3K, but not p38 MAPK, prevents the PGE2-mediated inhibition of eosinophil chemotaxis. Purified eosinophils were pretreated with SB-202190 (2 μM; A), LY-294002 (20 μM; B) or the respective vehicle at 37°C for 20 min and then mixed with PGE2 (3–100 nM), and loaded into the upper wells of a 48-well micro-Boyden chemotaxis chamber. Cells were allowed to migrate toward eotaxin and responses were expressed as a percentage of the control response to eotaxin. Data are shown as mean ± SE; n = 4–5; *, p < 0.05 vs vehicle.
end, eosinophils were pretreated with the respective vehicle, the p38 MAPK inhibitor SB-202190 (2 μM) or the PI3K inhibitor LY-294002 (20 μM) at 37°C for 20 min and then mixed with PGE2 (10–100 nM). As compared with its vehicle, SB-202190 was unable to prevent the PGE2-induced abrogation of eosinophil chemotaxis toward 1 nM eotaxin (Fig. 4A). In contrast, LY-294002 slightly reduced eosinophil migration toward eotaxin by itself and almost completely prevented the inhibitory effect of PGE2 (Fig. 4B). Similarly, pretreatment of eosinophils with the nonselective protein kinase inhibitor staurosporine (1 μM) or the selective PKC inhibitor chelerythrine (10 μM) at 37°C for 10 min completely prevented the inhibitory effect of PGE2 on eotaxin-induced migration (Fig. 5A). The EP4 antagonist ONO-AE3–208 (30 nM) at 37°C for 10 min completely prevented the inhibitory effect of low concentrations (1–10 nM), but not higher concentrations (30–100 nM), of PGE2. In agreement with the inhibitory effect of the EP2 antagonist, eosinophil migration toward eotaxin was inhibited by the EP2-selective agonists butaprost (0.1–1000 nM) and its demethylated analog butaprost-DM (0.01–100 nM) (38) with a potency 10- to 30-fold higher than that of PGE2 (Fig. 6A). The EP3 agonist ONO-AE-248 did not mimic the inhibitory effect of PGE2, whereas the EP4 agonist ONO-AE1–329 was capable of blunting the migration of eosinophils toward eotaxin but was at least 30 times less potent than butaprost (Fig. 6B).

In additional experiments we addressed the role of EP2 receptors in eosinophil degranulation. In fact, we observed that the C5a-induced release of EPO was significantly attenuated by both PGE2 and butaprost (30 nM; Fig. 7), although butaprost was less effective than PGE2 in this respect. Moreover, we observed that the EP4 agonist ONO-AE1–329 at equimolar concentrations was as effective as butaprost in inhibiting eosinophil degranulation. These data suggested that both EP2 and EP4 receptors can mediate the inhibitory effects of PGE2 on human eosinophils, with EP2 receptors being more important for the negative regulation of eosinophil migration.

**EP2 agonists inhibit eosinophil release from bone marrow**

The rapid release of eosinophils from the bone marrow is an important prerequisite of the accumulation of eosinophils in the tissue at sites of inflammation. The evasion of eosinophils from the bone marrow sinuses is believed to be governed by chemotactants such as eotaxin or PGD2 and by growth factors such as IL-5 (39, 40). The possible inhibitory effect of PGE2...
and EP2 receptors on the release of eosinophils from bone marrow was investigated in the in situ perfused hind limb of the guinea pig. Infusion of the selective CRTH2 receptor agonist 13,14-dihydro-15-keto-PGD2 (30 nM) or IL-5 (50 nM) into the hind limb caused a rapid increase of eosinophils in the effluent perfusate (Fig. 8, A and C) without having an effect on the release of other nucleated cells (Fig. 8, B and D). Concomitant infusion of PGE2 (30 nM), starting 10 min before the infusion of 13,14-dihydro-15-keto-PGD2 completely prevented the mobilization of eosinophils from bone marrow (Fig. 8A). Similarly, the mobilization of bone marrow eosinophils by IL-5 was significantly attenuated by PGE2 and even more profoundly by the EP2-selective PGE2 analog butaprost-DM (Fig. 8C).

**FIGURE 7.** EP2 and EP4 receptors mediate the inhibitory effect of PGE2 on eosinophil degranulation. Purified human eosinophils were pretreated with vehicle, the EP2 agonist butaprost, the EP4 agonist ONO-AE1-329, or PGE2 (30 nM each) for 5 min and then stimulated with C5a for 30 min at 37°C. The release of EPO activity into the supernatant was determined by photometry. Data were expressed as a percentage of the maximal control response (300 nM C5a) and are shown as the mean ± SE; n = 5–7; * p < 0.005 vs C5a alone (vehicle).

**FIGURE 8.** PGE2 and the EP2-selective agonist butaprost-DM attenuate the release of eosinophils from guinea pig femoral bone marrow. The isolated hind limb preparation was perfused with Krebs-Ringer solution, the effluent was collected in 10-min fractions and eosinophils and other nucleated cells in the effluent were enumerated. Infusion of 13,14-dihydro-15-keto-PGD2 (DK-PGD2; 30 nM) or IL-5 (50 pM) for 30 min (**) caused a rapid increase in eosinophil numbers in the effluent (A and C) while the number of other nucleated cells in the perfusate remained largely unchanged (B and D). Infusion of PGE2 or butaprost-DM (30 nM), which was started 10 min before that of DK-PGD2 or IL-5, respectively, markedly depressed the eosinophil mobilization. Data are shown as mean ± SE; n = 8–9; * p < 0.05 vs control.

**EP2 agonist inhibits allergen-induced accumulation of eosinophils in lung**

Our data suggested that PGE2 acting through EP2 receptors is a potent inhibitor of the in vitro migration of eosinophils and the rapid mobilization of eosinophils from the bone marrow. To investigate whether an EP2 agonist, butaprost, was also able to prevent the allergen-induced accumulation of eosinophils in the airways in vivo, mice were sensitized to OVA, injected daily with 50 µg of butaprost or vehicle s.c., and challenged with OVA by aerosol. An additional group of mice that were sensitized to OVA but received no aerosol challenge served as control. Thereafter, BAL was performed and eosinophil numbers in the fluid were analyzed by flow cytometry. In additional groups of mice (n = 3 each), lungs were not subjected to lavage but were processed for histology. Immunochemistry revealed that EP2 receptors were predominantly expressed in bronchial epithelial cells and in some of the few inflammatory cells present in control lungs (Fig. 9, A and B). In addition to epithelial cells, we observed profound EP2 staining in the infiltrating leukocytes (most likely eosinophil) in the lungs of allergen-challenged mice (Fig. 9, C and D). Similarly, human eosinophils stained positive for EP2 when analyzed by flow cytometry (Fig. 9G). Preabsorption of the Ab with EP2-blocking peptide completely prevented the staining of lungs of allergen-challenged mice (Fig. 9, D and E) and also that of human eosinophils (Fig. 9G), hence proving specificity of the EP2 staining. In the BAL fluid, allergen challenge caused a 3-fold increase in total leukocyte numbers as compared with control animals (Fig. 10). Excess leukocytes in the BAL fluid were identified as being eosinophils, while the numbers of other leukocytes did not change to a significant extent. Consequently, the frequency of eosinophils in the BAL fluid increased >10-fold from 2.4 ± 1.0% to 27.9 ± 7.3% (p < 0.05, n = 8) in control vs challenged.
mice, respectively. Treatment of mice with butaprost completely prevented the allergen challenge-induced increase in total leukocyte numbers and eosinophil counts in the BAL fluid (Fig. 10). This observation was reflected by a decrease of the frequency of eosinophils after allergen challenge form 27.9% to 9.8% (p < 0.05, n = 8) in animals treated with the vehicle or butaprost, respectively (Fig. 10). The absolute numbers of other leukocytes was unchanged by butaprost treatment with the vehicle or butaprost, respectively (Fig. 10). The absolute numbers of other leukocytes was unchanged by butaprost treatment with the vehicle or butaprost, respectively (Fig. 10).

**Discussion**

In this study we describe that the chemotactic responsiveness of isolated human eosinophils is restrained by low concentrations of PGE2 in vitro. The PGE2-induced inhibition of migration is brought about mainly by EP2 receptors and involves PI3K and PKC. Consistent with this notion, we show in animal models that EP2 agonists are capable of ameliorating the mobilization of eosinophils from bone marrow and eosinophil recruitment to sites of allergic inflammation. Therefore, our data provide a novel mechanistic concept to substantiate previous observations that exogenous PGE2 negatively controls eosinophil recruitment to sites of allergic inflammation (19–21), while inhibition of endogenous PGE2 biosynthesis in COX-1 or COX-2 knockout mice or after treatment with selective COX-1 or COX-2 inhibitors exaggerates the eosinophil infiltration in response to allergen (23–25).

The inhibitory effect of PGE2 on eosinophil recruitment seems to occur directly at the subcellular level; PGE2 very potently (IC50 ~ 3 nM) inhibited the migratory responsiveness of eosinophils, as chemotaxis toward three different classes of chemoattractants, i.e., eotaxin, PGD2, and C5a, was abrogated alike. Eosinophil migration was also markedly inhibited in the presence of activated monocytes, which are important sources of PGE2 production in vivo. In fact, LPS treatment of monocytes resulted in concentrations of ~3 nM PGE2 in the supernatants, which corresponded to the IC50 of exogenously added PGE2. Inhibition of COX in monocytes abrogated both the biosynthesis of PGE2 and the inhibition of eosinophil migration. These observations, hence, demonstrate that the inhibitory action of PGE2 might also bear physiological relevance.

The effects of PGE2 are mediated via four rhodopsin-type G protein-coupled receptors termed EP1, EP2, EP3, and EP4 (41). Expression of EP2 and EP4 receptors in eosinophils has been demonstrated at mRNA and protein levels, whereas data on EP1 and EP3 receptor expression have remained inconclusive (42, 43). Functionally, stimulation of EP4 but not EP2 receptors inhibits leukotriene C4 biosynthesis in human eosinophils (42). Our data show that PGE2 inhibition of eosinophil responsiveness toward chemoattractants is mediated mainly by EP2 receptors, as this was mimicked by the EP2-selective agonists butaprost and its demethylated analog with considerably higher potency than with PGE2 itself. This notion was corroborated by the observation that the nonselective EP1/EP2/EP3 antagonist AH6809, which has very
low affinity for EP4 receptors, completely blocked the inhibitory effect of PGE2 on eosinophil migration, while the EP4 antagonist was only partially effective. The involvement of EP1 and EP3 receptors could be ruled out, as the selective EP1 agonist SC-51089 was without effect on PGE2 inhibition of eosinophil migration, and an EP3-selective agonist did not mimic the inhibitory effect of PGE2. However, EP4 receptors also seem to be capable of conferring an inhibitory signal upon eosinophil migration, because an EP4 agonist likewise decreased the migratory responsiveness of eosinophils in chemotaxis assays. Interestingly, PGE2 and both the EP2 and EP4 agonists inhibited eosinophil degranulation as measured by C5a-induced EPO release, which might add to the anti-inflammatory effects of PGE2 in vivo.

Stimulation of EP2 or EP4 receptors usually increases intracellular cAMP levels. Unexpectedly, the inhibitory effect of PGE2 on eosinophil migration does not seem to be integrally dependent on activation of the adenyl cyclase pathway, because neither the adenyl cyclase inhibitor SQ-22536 nor the PKA inhibitor H-89 could prevent the inhibitory effect of PGE2. In detail, both cAMP inhibitors slightly reduced the inhibitory effect of PGE2 on eosinophil migration toward eotaxin, but this was not the case when PGD2 was used as the chemoattractant. Therefore, it seems that cAMP plays a major role in eosinophil migration toward eotaxin per se in agreement with the differential roles that cAMP plays in the complex process of leukocyte migration, depending on the subcellular localization of cAMP production and the relative expression level of different cAMP effectors such as PKA or Epac-1 (exchange protein directly activated by cAMP 1). As recently reviewed by Lorenowicz et al. (44), activation of PKA results in inhibition of cell surface expression of selectins and integrins and integrin activation, thereby resulting in reduced adhesion; but PKA is necessary for the clustering of integrins, which is a prerequisite for stable, sustained adhesion. Furthermore, PKA inhibits RhoA, a critical regulator of actin-based contractility. In contrast, cAMP also activates Epac-1, which mediates the activation of integrins locally at the leading edge and therefore facilitates leukocyte adhesion and motility. These findings hence might explain how both stimulation and inhibition of cAMP biosynthesis might have a negative impact on leukocyte migration. However, cAMP seems to play a smaller role in PGD2-induced migration, because inhibition of the cAMP pathway did not attenuate eosinophil chemotaxis toward PGD2 in the absence of PGE2. One explanation for this apparent discrepancy might be the fact that the eotaxin receptor, CCR3, signals through G1 proteins, leading to inhibition of adenyl cyclase, whereas the PGD2 receptor, CRTH2, signals through both G1 and Gq in eosinophils (30). Therefore, disruption of a major signaling pathway by lowering cAMP levels at baseline might cause more profound alterations in the well-coordinated subcellular events that govern the migration of the eosinophil in the case of eotaxin, as compared with PGD2.

Apart from increasing cAMP biosynthesis, a downstream event following stimulation of EP2 receptors is activation of PI3K (45, 46). Moreover, activation of PI3K can eventually lead to PKC activation (47, 48). Therefore, we investigated the effect of the PI3K inhibitor LY-294002 on eosinophil migration in the presence of PGE2. In agreement with previous findings, LY-294002 slightly inhibited eosinophil migration to eotaxin in the absence of PGE2, but almost completely prevented the inhibitory effect of PGE2. Moreover, exposure of eosinophils to the broad-spectrum protein kinase inhibitor staurosporine and the selective PKC inhibitor chelerythrine likewise prevented the PGE2-induced inhibition of eosinophil chemotaxis. The PKC activator PMA very potently (0.1 nM) mimicked the inhibitory effect of PGE2 on eosinophil chemotaxis, and this effect was also prevented by staurosporine or chelerythrine. In contrast, LY-294002 did not prevent the inhibitory effect of PMA on eosinophil migration. These data demonstrated that PI3K acting up-stream of PKC might be involved in the inhibitory action of PGE2 (49). Different PKC subtypes have been shown to modulate eosinophil functions, such as chemotaxis, degranulation, or adhesion, and increased expression and activation of eosinophil PKCz was recorded after allergen challenge (50).

Recently, p38 MAPK has been hypothesized to be essential for neutrophil orientation in opposing gradients of chemocompounds (51). Furthermore, we described that the MAPK pathway determines the hierarchy of eosinophil chemocompounds (11). In that study we observed that pretreatment of eosinophils with the chemocompounds eotaxin or 5-oxo-6,8,11,14-eicosatetraenoic acid inhibited the subsequent migration of the eosinophils toward PGD2 and that inhibition of p38 MAPK prevented this inhibitory effect and even converted it to enhancement of eosinophil migration. This pathway, however, does not seem to be involved in PGE2 inhibition of eosinophil migration because SB-202190, a highly selective inhibitor of the α- and β-isomers of p38 MAPK (52), failed to reverse the PGE2-induced inhibition of eosinophil migration.

It has been suggested previously that PGE2 also inhibits neutrophil chemotaxis with an IC50 of 90 nM (53). In agreement with that, PGE2 at concentrations up to 30 nM did not attenuate neutrophil migration toward IL-8 and C5a in the present study, whereas the inhibition of eosinophil migration was near maximal at that concentration. In contrast to its inhibitory effects in eosinophils and neutrophils, recent studies suggested that PGE2 enhances the chemotactic responsiveness of human monocytes and dendritic cells through activation of the EP2 and EP4 receptors (54, 55). It should be noted, however, that these effects were observed only at concentrations of PGE2 at least 10-fold higher than those in the present study.

The numbers of eosinophils at sites of inflammation depend on the rate of their release into the circulation from the bone marrow and the rate of their extravasation into the tissue. Only a few inflammatory mediators have been described to regulate eosinophil release from the bone marrow, including IL-5, eotaxin and PGD2 (33, 39, 40). In the present study we found that PGE2 via EP2 receptors also controls agonist-induced rapid mobilization of eosinophils from bone marrow. In the in situ perfused guinea pig hind limb, 30 nM PGE2 completely prevented the mobilization of eosinophils in response to infusion of the selective CRTH2 agonist 13,14-dihydro-15-keto-PGD2 and significantly attenuated the IL-5 induced eosinophil release. The EP2-selective agonist butaprost-DM showed even higher efficacy at the same concentration, suggesting that the EP2 receptor was involved.

Administration of PGE2 to humans and experimental animals has been shown to inhibit allergen-induced bronchoconstriction and airway eosinophilia (19, 20). Using immunohistochemistry of murine lung tissue we observed that EP2 receptor expression was confined to the epithelium in control lungs. After exposure to allergen, infiltrating leukocytes, presumably eosinophils, were also EP2 positive. EP2 expression was also confirmed in human peripheral blood eosinophils by using flow cytometry. Because stimulation of EP2 receptors attenuates the mobilization of eosinophils from bone marrow and eosinophil migration, we assessed the effect of the EP2 agonist butaprost on allergen-induced airway eosinophilia in mice sensitized to OVA. Butaprost almost completely abolished the allergen-induced increase in eosinophils in the BAL fluid, although it had no significant effect on blood eosinophilia after repeated allergen challenge. Nevertheless, this observation
does not preclude that butaprost inhibits the allergen-induced mobilization of eosinophils from bone marrow, but its concomitant inhibitory effect on eosinophil extravasation seems to compensate for the reduced rate of the bone marrow supply of eosinophils.

Taken together, in the present study we have described for the first time that PGE2 directly controls eosinophil migration on the cellular level and is highly potent and efficacious in this respect. We have dissected the receptors, the cellular effectors mechanisms, and the biochemical pathways behind these PGE2 effects. In detail, we have shown in vitro that eosinophil locomotion is attenuated mainly via EP2 receptors involving PI3K- and PKC-dependent pathways. Furthermore, using in vivo models we could show that EP2 agonists can attenuate the release of eosinophils from bone marrow and prevent the allergen-induced recruitment of eosinophils into the lungs. Previous studies have shown that PGE2 has bronchoprotective effects and also acts as an anti-inflammatory mediator in human airways (15, 19). However, the use of PGE2 as a therapeutic agent in asthma is hampered by major side effects such as acute bronchoconstriction, retrolental soresness, transient cough, and flu-like symptoms (15, 16, 19). Because these effects are thought to arise from EP1 or EP3 receptor stimulation, selective EP2 agonists might have a more favorable pharmacological profile as therapeutic options for allergic diseases. Moreover, activation of EP2 receptors protects against pulmonary fibrosis (22, 23). It is our experience that novel EP2 antagonists might have a more favorable pharmacological profile as therapeutic options for allergic diseases. Moreover, activation of EP2 receptors protects against pulmonary fibrosis (22, 23). The authors have no financial conflict of interest.

Disclosures

The authors have no financial conflict of interest.

References